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Effect of pH and level of concentrate in the diet on the production of biohydrogenation intermediates in a dual-flow continuous culture

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ABSTRACT

Milk fat depression in cows fed high-grain diets has been related to an increase in the concentration of *trans*-10 $C_{18:1}$ and *trans*-10,*cis*-12 conjugated linoleic acid (CLA) in milk. These fatty acids (FA) are produced as a result of the alteration in rumen biohydrogenation of dietary unsaturated FA. Because a reduction in ruminal pH is usually observed when high-concentrate diets are fed, the main cause that determines the alteration in the biohydrogenation pathways is not clear. The effect of pH (6.4 vs. 5.6) and dietary forage to concentrate ratios (F:C; 70:30 F:C vs. 30:70 F:C) on rumen microbial fermentation, effluent FA profile, and DNA concentration of bacteria involved in lipolysis and biohydrogenation processes were investigated in a continuous culture trial. The dual-flow continuous culture consisted of 2 periods of 8 d (5 d for adaptation and 3 d for sampling), with a 2 × 2 factorial arrangement of treatments. Samples from solid and liquid mixed effluents were taken for determination of total N, ammonia-N, and volatile fatty acid concentrations, and the remainder of the sample was lyophilized. Dry samples were analyzed for dry matter, ash, neutral and acid detergent fiber, FA, and purine contents. The pH 5.6 reduced organic matter and fiber digestibility, ammonia-N concentration and flow, and crude protein degradation, and increased nonammonia and dietary N flows. The pH 5.6 decreased the flow of $C_{18:0}$, *trans*-11 $C_{18:1}$ and *cis*-9, *trans*-11 CLA, and increased the flow of *trans*-10 $C_{18:1}$, $C_{18:2n-6}$, $C_{18:3n-3}$, *trans*-11,*cis*-15 $C_{18:2}$ and *trans*-10,*cis*-12 CLA in the 1 h after feeding effluent. The pH 5.6 reduced *Anaerovibrio lipolytica* (32.7 vs. 72.1 pg/10 ng of total DNA) and *Butyrivibrio fibrisolvens* vaccenic acid subgroup (588 vs. 1,394 pg/10 ng of total DNA) DNA concentrations. The high-concentrate diet increased organic matter and fiber digestibility, nonammonia and bacterial N flows, and reduced ammonia-N concentration and flow. The high-concentrate diet reduced *trans*-11 $C_{18:1}$ and *trans*-10 $C_{18:1}$, and increased $C_{18:2n-6}$, $C_{18:3n-3}$ and *trans*-10,*cis*-12 CLA proportions in the 1 h after feeding effluent. The increase observed in *trans*-10,*cis*-12 CLA proportion in the 1 h after feeding effluent due to the high-concentrate diet was smaller that that observed at pH 5.6. Results indicate that the pH is the main cause of the accumulation of *trans*-10 $C_{18:1}$ and *trans*-10, *cis*-12 CLA in the effluent, but the *trans*-10,*cis*-12 CLA proportion can be also affected by high levels of concentrate in the diet.
Key words: biohydrogenation, concentrate, polymerase chain reaction, pH

INTRODUCTION

Several theories have been proposed to explain milk fat depression, but the one that has gained support over the last decade suggests that mammary synthesis of milk fat is inhibited by unique *trans* fatty acids (**FA**) that are produced as a result of alterations in rumen biohydrogenation (Bauman and Griinari, 2003). A further examination of the isomer profile indicated that increases in ruminally derived *trans*-10 containing $C_{18:1}$ and $C_{18:2}$ isomers were more closely associated with milk fat depression than the general increase in total *trans*-$C_{18:1}$ or conjugated linoleic acid (**CLA**; Griinari et al., 1998; Griinari and Bauman, 1999). However, *trans*-10 $C_{18:1}$ does not directly control milk fat synthesis, although it responds to dietary factors, and its concentration is negatively correlated with milk fat yield response in cows (Bernard et al., 2008). On the other hand, it has been demonstrated that *trans*-10 *cis*-12 CLA inhibits milk fat synthesis in dairy cows (Baumgard et al., 2000).

Griinari et al. (1998) demonstrated that two conditions were necessary to observe a diet-induced milk fat depression: the availability of dietary unsaturated FA and an altered rumen environment that leads to incomplete biohydrogenation. In low-fiber diets, the change in microbial fermentation is characterized by a decline in rumen pH and a shift in the rumen pattern of VFA (lower acetate and higher propionate proportions; Bauman and Griinari, 2003). Griinari et al. (1998)

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observed that a 20:80 forage to concentrate (**F:C**) diet supplemented with unsaturated fat (corn oil, 4% DM) decreased ruminal pH (pH <6.0) and increased *trans*-10 $C_{18:1}$ concentration in milk (2.90 vs. 0.70%) compared with a 50:50 F:C diet supplemented with the same amount of corn oil. Moreover, Kalscheur et al. (1997) observed that a 25:75 F:C diet reduced ruminal pH (5.83 vs. 6.13), and Piperova et al. (2002) observed that the same diet increased the flow of *trans*-10 $C_{18:1}$ to the duodenum (29.13 vs. 5.73 g/d) compared with a 60:40 F:C diet. Because a reduction in ruminal pH is usually observed when high-concentrate diets are fed, these 2 factors are confounded, and it is not clear if the main cause of the production of these fatty acids is the level of concentrate in the diet, ruminal pH, or both. Kalscheur et al. (1997) observed that the addition of buffer to high-concentrate diets increased ruminal pH (6.02 vs. 5.83), and Piperova et al. (2002) observed that the buffer addition reduced the flow of *trans*-10 $C_{18:1}$ to the duodenum (8.11 vs. 29.13 g/d), suggesting that pH was the factor causing the alteration of the rumen biohydrogenation pathway resulting in *trans*-10 $C_{18:1}$ accumulation. However, decreasing the F:C ratio from 65:35 to 35:65 did not result in a reduced ruminal pH (6.38 ± 0.12), whereas duodenal flow of *trans*-10 $C_{18:1}$ increased (35.4 vs. 4.04 g/d; Loor et al., 2004). It is evident that significant interaction must exist between dietary F:C and pH on rumen biohydrogenation of unsaturated FA. However, to our knowledge, there is no literature available in which both factors were studied simultaneously to evaluate their contribution to the flow of FA in continuous culture. Therefore, we conducted an experiment to study the effect of pH and level of concentrate in the diet on biohydrogenation in a dual-flow continuous culture.

MATERIALS AND METHODS

Apparatus and Experimental Design

Eight 1,320-mL dual-flow continuous culture fermenters (Hoover et al., 1976) were used in 2 replicated periods of 8 d (5 d of adaptation and 3 d for sample collection). On the first day of each period, all fermenters were inoculated with ruminal fluid obtained from a rumen-fistulated cow (600 kg of BW) fed a 60:40 F:C diet. Temperature (38.5°C) and liquid (10%/h) and solid (5%/h) dilution rates were maintained constant and fermentation conditions were monitored with the LabView Software (FieldPoint, National Instruments, Austin, TX). Anaerobic conditions were maintained by infusion of N_2 at a rate of 40 mL/min. Artificial saliva (Weller and Pilgrim, 1974) was continuously infused into the flasks and contained 0.4 g/L urea to simulate recycled N. Treatments were arranged in a 2 × 2 factorial design, the main factors being the pH (high = 6.4 vs. low = 5.6) and the F:C ratio in the diet [low concentrate (**LC**) = 70:30 F:C vs. high concentrate (**HC**) = 30:70 F:C]. Ruminal pH was controlled at pH 6.4 ± 0.05 or pH 5.6 ± 0.05 by automatic infusion of 3 *N* HCl or 5 *N* NaOH. Consumption of NaOH averaged 20.5 and 12.1 mL/d for HC and LC diets, respectively. Consumption of HCl averaged 14.4 and 32.6 mL/d for HC and LC diets, respectively. Diets (95 g of DM/d) were fed in equal portions to the fermenters 3 times per day. Diets were designed to be isoproteic and to provide equal amounts of total FA with a similar $C_{18:2}$ and $C_{18:3}$ composition (Table 1).

Table 1. Ingredient, chemical, and fatty acid composition of the 2 experimental diets (LC = low concentrate diet, HC = high concentrate diet)

	Diet	
Item	LC	HC
Ingredient, %		
Alfalfa hay	70.0	30.0
Corn	15.0	36.8
Barley	6.50	18.4
Soybean meal	5.00	12.3
Soybean oil	3.00	1.60
Linseed oil	—	0.55
Premix[1]	0.50	0.50
Chemical composition, %		
DM	90.0	88.8
NDF	31.6	21.6
ADF	21.7	12.0
NFC[2]	35.6	48.8
CP	18.1	17.5
Ether extract	5.42	5.14
Ash	9.33	6.93
Fatty acid, % of total FA		
$C_{16:0}$	15.5	14.2
$C_{18:0}$	4.00	3.22
$C_{18:1}$ *cis*-9	19.4	22.3
$C_{18:2}$ *cis*-9,*cis*-12	44.0	43.3
$C_{18:3}$ *cis*-9,*cis*-12,*cis*-15	9.03	10.5

[1]The premix contained (per kilogram of DM): 7 mg of Co, 167 mg of Cu, 33 mg of I, 2,660 mg of Mn, 27 mg of Se, 4,660 mg of Zn, 1,000 kIU of vitamin A, 200 kIU of vitamin D_3, 1,330 mg of vitamin E, 267 g of urea, 67 g of NaCl, 33 g of sulfur, and 300 mg of MgO.
[2]NFC = [100 – (ash + CP + NDF + ether extract)].

Sample Collection

During the last 3 d of each period, 60 mL each of solid and liquid effluents were collected at 1 h after the morning feeding to study effluent FA profile. Additionally, throughout the 8 d of each period, 1.5 mL of fermenter liquid was collected 1 h after the morning feeding to determine DNA concentrations of *Anaerovibrio lipolytica*, *Butyrivibrio fibrisolvens* stearic acid producer subgroup (***Butyrivibrio* SA**), and *Butyrivibrio fibrisolvens* vaccenic acid producer subgroup (***Butyrivibrio* VA**)

(Paillard et al., 2007a) using PCR analyses to study the adaptation process of the microorganisms to treatments. During sampling days, effluent collection vessels were maintained at 4°C to impede microbial action. Solid and liquid 24-h effluents were mixed and homogenized for 1 min, and a 500-mL sample was removed via aspiration. Upon completion of each period, effluents from the 3 sampling days were composited within fermenter and homogenized for 1 min. Samples were taken for the determination of total N, ammonia-N, and VFA concentrations, and the remainder of the sample was lyophilized. The DM content was determined from the lyophilized sample, which was further analyzed for ash, NDF, ADF, FA, and purine contents.

Bacterial cells were obtained from fermenter flasks on the last day of each experimental period. Briefly, 100 mL of a 2 g/L methylcellulose solution and small marbles (30 of 2-mm diameter and 15 of 4-mm diameter) were added to each fermenter and mixed for 1 h at 39°C to remove attached bacteria. After incubation, fermenter flasks were refrigerated for 24 h at 4°C and then agitated for 1 h to dislodge loosely attached bacteria. Finally, the fermenter contents were filtered through cheesecloth and washed with saline solution (8.5 g/L NaCl). Bacterial cells were isolated within 4 h by differential centrifugation at 1,000 × *g* for 10 min to separate feed particles, and the supernatant was centrifuged at 20,000 × *g* for 20 min to isolate bacterial cells. Pellets were rinsed twice with saline solution and centrifuged at 20,000 × *g* for another 20 min. The final pellet was recovered with distilled water to prevent contamination of bacteria with ash. Bacterial cells were lyophilized and analyzed for DM, ash, N, and purine contents. Digestion of OM, fiber and CP, and flows of total, nonammonia, bacterial, and dietary N were calculated as described by Stern and Hoover (1990).

Chemical Analyses

Feed samples, lyophilized effluent and bacteria were analyzed for DM (24 h at 103°C), ash (4 h at 550°C), and total N (Kjeldahl method; AOAC, 1995). Neutral and acid detergent fiber of feed and effluents were analyzed by the detergent system using sodium sulfite and a heat-stable α-amylase (Van Soest et al., 1991), and fat was analyzed by Soxhlet after acid hydrolysis (AOAC, 1995).

Samples for VFA were prepared as follows (Jouany, 1982): 1 mL of a solution comprising 0.2% (wt/wt) of mercuric chloride, 0.2% (wt/wt) 4-methylvaleric acid (as an internal standard), and 2% (vol/vol) orthophosphoric acid was added to 4 mL of filtered rumen fluid and frozen. Samples were centrifuged at 3,000 × *g* for 30 min, and the supernatant fluid was analyzed by GC (model 6890, Hewlett Packard, Palo Alto, CA) using a polyethylene glycol nitroterephtalic acid-treated capillary column (BP21, SGE, Europe Ltd., Buckinghamshire, UK) at 275°C in the injector and at a gas flow rate of 29.9 mL/min. Ammonia-N was analyzed in a 4-mL sample of filtered fermenter fluid that was acidified with 4 mL of 0.2 *N* HCl and frozen. Samples were centrifuged at 15,000 × *g* for 15 min, and the supernatant was analyzed by colorimetry (UV-120-01, Shimadzu, Kyoto, Japan) for ammonia-N (Chaney and Marbach, 1962). Samples of lyophilized effluent and bacterial cells were analyzed for purine content (adenine and guanine) by HPLC (Beckman Instruments, Palo Alto, CA) as described by Balcells et al. (1992), using allopurinol as an internal standard.

The 1-h postfeeding effluent samples of d 6 to 8 were composited by fermenter. Lipid extraction from the lyophilized effluents and diets was performed as described by Folch et al. (1957) with some modifications as described by Chow et al. (2004). Methylation of FA was performed as described by Raes et al. (2001). The fatty acid methyl esters were extracted twice with 2 mL of hexane, and pooled extracts were evaporated to dryness under N_2 gas. The residue was dissolved in 1 mL of hexane and analyzed by GC (HP 6890, Brussels, Belgium) on a CP-Sil88 column for fatty acid methyl esters (100 m × 250 μm × 0.2 μm, Chrompack, Middelburg, the Netherlands; Raes et al., 2001). The GC conditions were injector: 250°C; detector: 280°C; H_2 as carrier gas; temperature program: 150°C for 2 min, followed by an increase of 1°C/min to 200°C, then 1°C/min to 215°C. Peaks were identified by comparing the retention times with those of the corresponding standards (Sigma, Bornem, Belgium, and Nu-Chek Prep, Elysian, MN).

DNA Extraction and Quantification

Bacterial cultures and rumen fluid DNA samples of d 1, 3, and 5 of adaptation and a composited sample on d 6 to 8 were extracted by physical disruption using a bead-beating method (Mini-Beater; Biospec Products, Bartlesville, OK) according to the method described by Whitford et al. (1998) with modifications of M. Blanch (Universidad Autónoma de Barcelona, Barcelona, Spain; personal communication). Concentration of DNA was measured by spectrophotometry (NanoDrop ND-1000 Spectophotometer, Nanodrop Technologies, Wilmington, DE).

PCR Analyses

The primers set used for PCR is described in Table 2. *Anaerovibrio lipolytica* specific primers were designed

Table 2. Description of forward (F) and reverse (R) primers used in the amplification of *Anaerovibrio lipolytica*, *Butyrivibrio* stearic acid (SA) producer subgroup, *Butyrivibrio* vaccenic acid (VA) producer subgroup, and universal bacteria

Target	Primer sequence (5′ to 3′)	Product size, bp
Anaerovibrio lipolytica	F-TTG GGT GTT AGA AAT GGA TTC TAG TG R-TCG AAA TGT TGTCCC CAT CTG	82
Butyrivibrio SA	F-TGA AAA ACT CCG GTG GTA TGA GAT R- CCG TGT CTC AGT CCC AAT GTG	126
Butyrivibrio VA	F-TGC ATT GGA AAC TGT AGA ACT AGA GTG T R-GCG TCA GTA ATC GTC CAG TAA GC	124
Universal bacteria	F-AGA GTT TGA TCC TGG CTC AGG A R-TGC TGC CTC CCG TAG GAG T	—

from 16S ribosomal DNA sequence available in the GeneBank database (AB034191) using the Primer Express Software (Applied Biosystems, Warrington, UK), the BLAST program at the National Center for Biotechnology Information site, and the CLUSTALW program at the European Bioinformatic Institute site were used to ensure specificity of primers. *Butyrivibrio* SA and VA subgroup-specific primers were modified from those described by Paillard et al. (2007b) and R. J. Wallace (Rowett Research Institute, Aberdeen, UK; personal communication), respectively, to adapt them to our PCR conditions. For universal bacteria, previously published primers EUB008 and EUB338 (Hicks et al., 1992) were adapted for our PCR conditions. Conditions of PCR were 50°C for 2 min; 95°C for 10 min; 35 cycles of 95°C for 15 s; and 60°C for 1 min. Each conventional PCR mixture (20 μL) contained (final concentrations) 1 × Power SYBR Green PCR Master Mix (Applied Biosystems), 0.5 μ*M* each primer, and 10 ng of genomic DNA (100 ng of genomic DNA for *A. lipolytica*). The PCR were performed in an ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems) using optical-grade 96-well plates. To construct a calibration curve and to determine the PCR efficiency and sensibility, serial dilutions (1/10) of DNA prepared from pure cultures of *A. lipolytica* (ATCC 33276), *Butyrivibrio* sp. (NCFB 2249), and *Butyrivibrio* sp. (NCFB 2434) were used. The standard curve of *A. lipolytica* had a slope of −3.417 and a regression coefficient of 0.997, that of *Butyrivibrio* SA subgroup had a slope of −3.219 and a coefficient of 0.997, and that of *Butyrivibrio* VA subgroup had a slope of −3.499 and a regression coefficient of 0.991. Efficiencies of PCR, calculated as described by Ginzinger (2002), were 96.2, 104.5, and 93.1% for *A. lipolytica*, *Butyrivibrio* SA subgroup, and *Butyrivibrio* VA subgroup, respectively.

Statistical Analyses

Results of DM, OM, NDF, ADF, and CP digestibilities, VFA, ammonia-N concentrations, effluent FA profile, and flows of total, nonammonia, bacterial, and dietary N, and PCR results of the composite samples on d 6 to 8 were analyzed as a randomized complete block design. Three DM values from the first period were omitted from the analyses because of problems with lyophilization. The 2 periods were considered as blocks, and each individual fermenter served as the experimental unit. Results were analyzed by the PROC MIXED procedure of SAS (v. 9.1, SAS Institute, Cary, NC), using the following model:

$$Y_{ij} = \mu + B_i + C_j + P_k + (C \times P)_{jk} + e_{ijk},$$

where μ was the mean, B was the period as block, C and P were the concentrate level and the pH effects, and $C \times P$ was the concentrate level by pH interaction. Results of the effect of treatments on microbial adaptation were analyzed using PROC MIXED for repeated measures (Littell et al., 1998) using the following model:

$$Y_{ijk} = \mu + B_i + C_j + P_k + D_l + (C \times P)_{jk} + (C \times D)_{jl} + (P \times D)_{kl} + e_{ijkl},$$

where μ was the mean; B was the period as block; C, P, and D were the concentrate level, pH, and day effects; and $C \times P$, $C \times D$, and $P \times D$ were the concentrate level by pH, the concentrate level by day, and pH by day interactions, respectively. The interaction $C \times P \times D$ was also tested in the model and in the cases where it was not significant, it was removed from the model. The only variable where it was significant was in *A. lipolytica* DNA concentration. Results were subjected to 3 covariance structures: compound symmetric, autoregressive order one, and unstructured covariance. The covariance structure that yielded the lowest Schwarz's Bayesian criterion (autoregressive order one for *Butyrivibrio* SA and *Butyrivibrio* VA subgroup, and compound symmetric for *A. lipolytica*) was considered the most desirable analysis. Orthogonal constrants were used to determine

Table 3. Effect of level of concentrate in the diet (LC: low concentrate vs. HC: high concentrate) and pH (6.4 vs. 5.6) on true OM and fiber digestion, and on total VFA concentration and profile in continuous culture

	LC		HC			*P*-value[1]		
Item	6.4	5.6	6.4	5.6	SEM	C	P	C × P
Digestion, %								
True OM	48.3	44.7	56.0	50.8	2.9	0.002	0.019	0.613
NDF	32.5	16.0	49.4	22.9	2.7	0.002	<0.001	0.105
Total VFA, m*M*	119[b]	91.0[c]	134[a]	116[b]	3.3	<0.001	<0.001	0.013
BCVFA,[2] m*M*	2.63[b]	0.58[c]	3.92[a]	0.60[c]	0.23	<0.001	<0.001	<0.001
VFA, mol/100 mol								
Acetate	64.9	54.4	57.1	45.2	1.3	<0.001	<0.001	0.377
Propionate	19.0[d]	28.5[b]	21.7[c]	36.7[a]	0.74	<0.001	<0.001	<0.001
Butyrate	11.4[c]	12.6[bc]	15.5[a]	13.4[b]	0.55	<0.001	0.401	0.007
Valerate	2.46	3.85	2.72	4.18	0.10	0.007	<0.001	0.713
Acetate:propionate	3.43	1.91	2.64	1.24	0.083	<0.001	<0.001	0.428

[a–d]When interaction is significant ($P < 0.05$), different superscripts within a row indicate means differ significantly.

[1]C = concentrate level, P = pH, and C × P = concentrate by pH interaction.

[2]BCVFA = branched-chain VFA.

if day had linear or quadratic effects on the parameter used. Results are reported as least squares means. Significance and tendencies were declared at $P < 0.05$ and $P < 0.10$, respectively, unless otherwise indicated.

RESULTS AND DISCUSSION

Ruminal Fermentation

Digestion of OM and fiber, total VFA and branched-chain VFA (**BCVFA**) concentrations, acetate proportion and the acetate to propionate ratio were lower and propionate and valerate proportions were higher at pH 5.6 than at pH 6.4 (Table 3). Ammonia-N concentration and flow and CP degradation were lower at pH 5.6 than at pH 6.4, and bacterial N flow tended to be also lower at pH 5.6 than at pH 6.4 (Table 4). Nonammonia and dietary N flows were higher at pH 5.6 than at pH 6.4. These results agree with the reported effects of low pH on rumen microbial fermentation (Shriver et al., 1986; Cerrato-Sánchez et al., 2007; Calsamiglia et al., 2008). Fibrolytic bacteria are sensitive to low pH (Russell and Dombrowski, 1980) resulting in reduced OM and fiber digestion and changes in the VFA profile observed. Undigested fiber within feed could have reduced the access of bacteria and enzymes to the protein (Wallace and Cotta, 1989; Devant et al., 2000), leading to the observed increase in dietary N flow and reduced CP degradation and ammonia-N concentration. The efficiency of microbial protein synthesis was not affected by pH (Table 4), and the tendency to reduce the bacterial N flow at pH 5.6 was attributed to a reduction in OM digestion (Table 3).

True OM and fiber digestion were higher in the HC than in the LC diet (Table 3). Under in vivo conditions, decreasing the F:C ratio did not modify true OM or

Table 4. Effect of level of concentrate in the diet (LC: low concentrate vs. HC: high concentrate) and pH (6.4 vs. 5.6) on N metabolism of rumen microbes in continuous culture

	LC		HC			*P*-value[1]		
Item	6.4	5.6	6.4	5.6	SEM	C	P	C × P
N-NH_3, mg/100 mL	16.4	8.39	12.9	2.30	1.5	0.008	<0.001	0.397
N flow, g/d								
Ammonia	0.52	0.27	0.41	0.08	0.046	0.007	<0.001	0.401
Nonammonia	2.64	2.90	2.76	3.04	0.054	0.038	0.001	0.909
Dietary	1.24	1.65	1.12	1.51	0.16	0.288	0.008	0.955
Bacterial	1.48	1.25	1.64	1.54	0.16	0.030	0.081	0.481
CP degradation,[2] %	54.9	39.8	57.8	42.9	6.0	0.496	0.007	0.985
EMPS,[3] g/kg of OMTD	35.3	32.3	33.0	34.1	2.0	0.883	0.547	0.242

[1]C = concentrate level, P = pH, and C × P = concentrate × pH interaction.

[2]CP degradation = [(g of dietary N intake – g of effluent dietary N)/g of dietary N intake] × 100.

[3]EMPS = efficiency of microbial protein synthesis [g of N/kg of OM truly digested (OMTD)].

fiber digestibilities (Hussein et al., 1995; Ueda et al., 2003) except when ruminal pH was reduced (Kalscheur et al., 1997). Using dual-flow continuous cultures, Rodríguez-Prado et al. (2004) and Calsamiglia et al. (2008) observed that the concentrate content of the diet did not affect true OM and fiber digestion. All these results suggest that the frequently observed lower OM and fiber digestion with high-concentrate diets is due to an effect of pH rather than a direct effect of the concentrate content of diet. Acetate proportion and the acetate to propionate ratio were lower, and valerate proportion was higher in the HC than in the LC diet (Table 3) consistent with previous in vitro and in vivo reports (Ueda et al., 2003; Rodríguez-Prado et al., 2004).

There was a significant interaction between diet and pH for total VFA and BCVFA concentrations, and propionate and butyrate proportions. Total VFA concentration was higher in HC than in LC, and the concentration of the HC diet at pH 5.6 was similar to that of the LC diet at pH 6.4 (Table 3). This is consistent with the higher OM digestion in HC than in LC diets, and with the numerical similar OM digestion of the HC diet at pH 5.6 and the LC diet at pH 6.4 (Table 3). Concentration of BCVFA was higher in HC than in LC diet at pH 6.4 and was similar between diets at pH 5.6. Russell et al. (1991) observed that amino acids can be incorporated directly into bacteria without deamination if energy is available, which would explain the lower ammonia-N concentration and flow observed in HC diet (Table 4). A lower deamination in the HC diet would result in lower concentrations of BCVFA; however, the opposite was observed in the current experiment (Table 3). The highest propionate proportion was observed in the HC diet at pH 5.6, followed by LC diet at pH 5.6, HC diet at pH 6.4, and LC diet at pH 6.4. This agrees with Calsamiglia et al. (2008) who observed that propionate concentration was the result of the combined effects of pH and diet and that the maximal propionate production was observed at pH 5.5. Butyrate proportion was higher in HC than in LC diet at pH 6.4, and it was similar between the 2 diets at pH 5.6 (Table 3). The higher butyrate proportion observed in the HC diet compared with the LC diet at pH 6.4 is in contrast with Rodríguez-Prado et al. (2004) and Calsamiglia et al. (2008) who observed no effect of the level of concentrate in the diet on butyrate proportion.

The HC diet resulted in lower ammonia-N concentration and flow than the LC diet (Table 4) and agrees with Calsamiglia et al. (2008) who also observed a lower ammonia-N concentration and flow in a 10:90 F:C than in a 60:40 F:C ratio diet. The lower ammonia-N concentration and flow in HC than in LC diet could be explained because the HC diet favored greater use of ammonia for the de novo synthesis of amino acids that were incorporated in bacterial protein, which would agree with the higher nonammonia and bacterial N flows observed in the HC compared with the LC diet (Table 4). However, efficiency of microbial protein synthesis was not affected by the concentrate content of diet (Table 4), probably because the HC diet also had higher true OM digestion (Table 3).

Biohydrogenation Intermediates

The proportions of $C_{18:0}$, *trans*-11 $C_{18:1}$, *cis*-9,*trans*-11 CLA, and *trans*-10,*cis*-12 CLA were lower and that of *trans*-10 $C_{18:1}$, *cis*-9 $C_{18:1}$, $C_{18:2n-6}$, $C_{18:3n-3}$, and *trans*-11,*cis*-15 $C_{18:2}$ higher at pH 5.6 than at pH 6.4 in the 24-h effluent (Table 5). The higher proportion of *cis*-9 $C_{18:1}$, $C_{18:2n-6}$, and $C_{18:3n-3}$ reflects the inhibition of *cis*-9 $C_{18:1}$, $C_{18:2n-6}$, and $C_{18:3n-3}$ apparent biohydrogenation at low pH that has been observed before (Van Nevel and Demeyer, 1996; Qiu et al., 2004). The lower proportion of *cis*-9,*trans*-11 CLA and *trans*-11 $C_{18:1}$ at low pH was also observed in batch cultures by Troegeler-Meynadier et al. (2003). The lower *trans*-11 $C_{18:1}$ and higher *trans*-10 $C_{18:1}$ proportions when pH decreased was previously reported by Griinari et al. (1998), who observed that increasing the concentrate contents of a diet containing unsaturated FA reduced ruminal pH and resulted in a lower proportion of *trans*-11 $C_{18:1}$ and a higher proportion of *trans*-10 $C_{18:1}$ in milk. Kalscheur et al. (1997) also observed that animals fed a high-concentrate diet (75% DM) had lower ruminal pH (5.83 vs. 6.14), and Piperova et al. (2002) observed that the same diet increased the duodenal flow of *trans*-10 $C_{18:1}$ (29.13 vs. 5.73 g/d) compared with animals fed a low-concentrate diet (40% DM). The pH 5.6 resulted in a 17-fold increase in *trans*-10 $C_{18:1}$, whereas *trans*-11 $C_{18:1}$ and $C_{18:0}$ effluent proportions decreased approximately 80% compared with pH 6.4. A similar effect was observed when incubating extruded soybean or linseed at pH 6.4 and 5.6 (Fuentes et al., 2008). *Trans*-10 $C_{18:1}$ is thought to arise from the hydrogenation of *trans*-10,*cis*-12 CLA (Bauman and Griinari, 2003). Nevertheless, in the current experiment the *trans*-10,*cis*-12 CLA proportion was lower at pH 5.6 compared with pH 6.4 in the 24-h effluent (Table 5). This is in contrast with in vivo (Piperova et al., 2002) and in vitro (Choi et al., 2005; AbuGhazaleh and Jacobson, 2007) results, in which *trans*-10,*cis*-12 CLA production was inversely related to pH. The effect of pH on effluent FA proportions at 1 h postfeeding (Table 5) were similar to the ones observed in the 24-h effluents (Table 5), except for *trans*-10, *cis*-12 CLA proportion, which was higher at pH 5.6 than at pH 6.4 at 1 h postfeeding (Table 5).

Table 5. Effect of level of concentrate in the diet (LC: low concentrate vs. HC: high concentrate) and pH (6.4 vs. 5.6) on 1-h and 24-h postfeeding effluent fatty acid profile (g/100 g of C_{18} fatty acids)

	LC		HC			*P*-value[2]		
Fatty acid profile[1]	6.4	5.6	6.4	5.6	SEM	C	P	C × P
1-h effluent								
$C_{18:0}$	47.8	11.8	48.5	10.2	3.2	0.872	<0.001	0.683
*t*10 $C_{18:1}$	1.82^{c}	27.1^{a}	0.35^{c}	18.3^{b}	2.9	0.027	<0.001	0.095
*t*11 $C_{18:1}$	5.71^{a}	1.06^{c}	3.21^{b}	0.39^{c}	0.46	0.008	<0.001	0.085
*c*9 $C_{18:1}$	14.1^{c}	20.6^{b}	15.8^{c}	24.9^{a}	0.74	0.012	<0.001	0.077
$C_{18:2n-6}$	17.7	27.4	18.9	31.8	1.2	0.049	<0.001	0.229
*t*11,*c*15 $C_{18:2}$	0.15	1.04	0.12	1.18	0.10	0.430	<0.001	0.268
*c*9,*t*11 CLA	0.11	0.03	0.07	0.004	0.024	0.175	0.005	0.693
*t*10,*c*12 CLA	0.14	0.47	0.17	0.60	0.033	0.022	<0.001	0.128
$C_{18:3n-3}$	3.53^{c}	5.11^{b}	4.97^{b}	7.92^{a}	0.33	<0.001	<0.001	0.064
24-h effluent								
$C_{18:0}$	59.3	15.5	62.1	9.5	3.5	0.602	<0.001	0.153
*t*10 $C_{18:1}$	1.86	31.4	0.76	23.6	3.4	0.137	<0.001	0.240
*t*11 $C_{18:1}$	6.97	1.71	4.04	0.69	0.63	0.012	<0.001	0.159
*c*9 $C_{18:1}$	11.0^{c}	18.6^{b}	12.0^{c}	24.7^{a}	0.61	0.001	<0.001	0.003
$C_{18:2n-6}$	8.01^{c}	19.3^{b}	8.95^{c}	27.9^{a}	1.6	0.013	<0.001	0.034
*t*11,*c*15 $C_{18:2}$	0.19	1.34	0.19	1.58	0.17	0.344	<0.001	0.347
*c*9,*t*11 CLA	0.98	0.11	0.78	0.01	0.18	0.402	0.001	0.774
*t*10,*c*12 CLA	0.68^{ab}	0.42^{bc}	0.90^{a}	0.22^{c}	0.11	0.940	0.002	0.085
$C_{18:3n-3}$	1.88^{c}	3.95^{b}	2.06^{c}	6.79^{a}	0.41	0.004	<0.001	0.008

[a–c]When interaction is significant ($P < 0.05$), different superscripts within a row indicate means differ significantly.

[1]*t* = *trans*; *c* = *cis*; CLA = conjugated linoleic acid.

[2]C = concentrate level, P = pH, and C × P = concentrate × pH interaction.

Wallace et al. (2007) also observed a reduction in *trans*-10,*cis*-12 CLA concentration after 4 h in 24-h in vitro incubations of linoleic acid with mixed ovine ruminal microorganisms.

The proportions of *cis*-9 $C_{18:1}$, $C_{18:2n-6}$, and $C_{18:3n-3}$ were higher and that of *trans*-11 $C_{18:1}$ lower in the HC than in the LC diet in the 1-h and 24-h effluents (Table 5). These results suggest that biohydrogenation is more active in LC than in HC diets because there is less proportion of biohydrogenation precursors (*cis*-9 $C_{18:1}$, $C_{18:2n-6}$, $C_{18:3n-3}$) and a greater proportion of biohydrogenation intermediates, mainly *trans* $C_{18:1}$, in the effluent. This is in agreement with Loor et al. (2004) who also observed that there was less biohydrogenation of $C_{18:2n-6}$ and $C_{18:3n-3}$ in diets rich in concentrate (65C:35F; 74.5 and 83.9%) compared with diets rich in forage (65F:35C; 77.7 and 89.9%) in vivo. There was a significant interaction between diet and pH for *cis*-9 $C_{18:1}$, $C_{18:3n-3}$, and $C_{18:2n-6}$ proportions in the 24-h effluent. The proportions of *cis*-9 $C_{18:1}$, $C_{18:3n-3}$, and $C_{18:2n-6}$ in the effluent were not affected by the level of concentrate in the diet at pH 6.4, whereas proportions of *cis*-9 $C_{18:1}$, $C_{18:3n-3}$, and $C_{18:2n-6}$ were higher in HC diet when pH was 5.6 (Table 5). This result would suggest that the biohydrogenation inhibition observed at low pH could partially be alleviated using higher levels of fiber in the diets. There was no effect of diet on *trans*-10,*cis*-12 CLA proportion in the 24-h effluent (Table 5). However, there was an effect of diet for its proportion in the 1-h postfeeding effluent (Table 5). The *trans*-10,*cis*-12 CLA proportion was higher in the HC diet than the LC diet at 1 h postfeeding. Several studies have demonstrated that increasing amounts of readily digestible carbohydrates in the diet are associated with increased proportions of *trans*-10, *cis*-12 CLA and *trans*-10 $C_{18:1}$ in milk fat (Kalscheur et al., 1997; Griinari et al., 1998; Piperova et al., 2000). However, Griinari et al. (1998) and Kalscheur et al. (1997) observed that diets rich in concentrate were accompanied by a lower ruminal pH. Because in vivo the change in pH occurs as a consequence of feeding high-concentrate diets, the observed results are confounded between the effects of rumen acidosis and the effects of dietary concentrate level. Results of the present experiment indicate that the pH is the main factor affecting the biohydrogenation process that results in the accumulation of *trans*-10 $C_{18:1}$ and *trans*-10,*cis*-12 CLA; however, *trans*-10,*cis*-12 CLA proportion in the effluent can also be affected by high levels of concentrate in the diet, but to a lesser extent.

DNA Concentrations

Changes in DNA concentrations of *A. lipolytica*, *Butyrivibrio* VA, and *Butyrivibrio* SA subgroups along adaptation days are presented in Table 6. The DNA

Table 6. Effect of level of concentrate in the diet (LC: low concentrate vs. HC: high concentrate) and pH (6.4 vs. 5.6) on quantification (pg/10 ng DNA) of *Anaerovibrio lipolytica* and *Butyrivibrio fibrisolvens* vaccenic acid (VA) and stearic acid (SA) producer subgroups during adaptation days

	LC		HC			*P*-value[1]		
Item	6.4	5.6	6.4	5.6	SEM	C	P	C × P
A. lipolytica								
d 1	96.7	50.8	103	141	49	0.030	0.839	0.055
d 3	47.6	30.0	31.2	27.5	12	0.233	0.181	0.373
d 5	50.0	4.37	97.9	3.45	24	0.107	0.001	0.096
Contrast[2]	L (0.057)	L (0.029)	Q (0.010)	L (0.001)				
B. fibrisolvens VA								
d 1	1,161	1,014	1,275	562	185	0.382	0.040	0.155
d 3	1,457	1,122	1,750	230	381	0.334	0.011	0.072
d 5	1,465[ab]	942[b]	1,820[a]	104[c]	326	0.320	0.001	0.026
Contrast	NS (0.348)	NS (0.620)	L (0.072)	L (0.001)				
B. fibrisolvens SA								
d 1	354	323	377	383	156	0.451	0.816	0.732
d 3	522	518	450	454	85	0.386	0.998	0.958
d 5	491	605	401	433	133	0.125	0.374	0.614
Contrast	NS (0.227)	NS (0.137)	NS (0.461)	NS (0.714)				

[a–c]When interaction is significant ($P < 0.05$), different superscripts within a row indicate that means differ significantly.

[1]C = concentrate level, P = pH, and C × P = concentrate × pH interaction.

[2]Contrast: L = linear, Q = quadratic, NS = not significant.

concentration of *A. lipolytica* declined linearly along adaptation days at pH 5.6 until almost disappearing, indicating that this bacteria is very sensitive to low pH conditions. Previous research also showed that growth of *A. lipolytica* and lipase activity was lowered at low pH (Hobson, 1965; Henderson et al., 1969). A decline in *A. lipolytica* DNA concentration was observed in the HC diet at pH 6.4 on d 3 after inoculation and there was a great increase in its DNA concentration 2 d later (i.e., d 5 after inoculation). We speculate that this temporary reduction in *A. lipolytica* DNA concentration could be an effect of the adaptation process of bacteria to a different diet and medium in the fermenters. It seems that 2 d are necessary to adapt ruminal bacteria to in vitro conditions, because DNA concentrations on d 5 of adaptation (Table 6) were similar to that observed on d 6 to 8 (Table 7). This result agrees with those of Cardozo et al. (2004) and Busquet et al. (2005) who, when evaluating the VFA profile, also observed that 2 d were necessary for the adaptation of ruminal microflora to the fermentation conditions in vitro. Regarding *B. fibrisolvens* VA subgroup, we observed a linear increase in its DNA concentration in the HC diet at pH 6.4. Choi et al. (2005) and Kim et al. (2003) also observed that biohydrogenation was more active with rumen bacteria from cows fed low-fiber compared with high-fiber diets using a similar F:C ratio (77:23) to the one used in the current experiment. Therefore, it seems that bacteria responsible for the biohydrogenation process are not affected by HC diets when a minimum level of fiber (about 30%) is guaranteed. The DNA concentration of *B. fibrisolvens* SA subgroup was little altered along experimental days (Table 7). This is in contrast with the accepted idea that cellulolytic bacteria are sensitive to low pH conditions. Although biohydrogenation was inhibited at pH 5.6 in the present experiment, it is also clear that other intermediates were produced at these low pH conditions, such as *trans*-10 $C_{18:1}$ and *trans*-10,*cis*-12 CLA, indicating that some bacteria can be resistant to low pH conditions and are capable of producing these intermediate *trans* FA.

The DNA concentrations of *A. lipolytica*, *Butyrivibrio* VA, and *Butyrivibrio* SA on d 6 to 8 are presented in Table 7. There was a significant interaction between diet and pH in DNA concentrations of *A. lipolytica* and *Butyrivibrio* VA subgroup (Table 7). The DNA concentration of *A. lipolytica* was reduced at pH 5.6 in both diets and its concentration at pH 6.4 was higher in the HC diet than in the LC diet. The reduced *A. lipolytica* DNA concentration observed at pH 5.6 would help to explain the higher $C_{18:2n-6}$ and $C_{18:3n-3}$ proportions in the effluent at pH 5.6 (Table 5). In relation to the higher *A. lipolytica* DNA concentration at pH 6.4 in the HC diet compared with the LC diet, Tajima et al. (2001) also observed an increase in *A. lipolytica* DNA concentration 3 d after a switch from a low- to a high-concentrate diet (0.137 vs. 0.440 mmol of 16S rDNA per milligram of total rumen DNA). Mackie et al. (1978) found an increased proportion of *A. lipolytica* in the rumen of sheep that were stepwise adapted to high-concentrate diets. In spite of the increased *A. lipolytica* DNA concentration in the HC diet at pH 6.4, amounts of dietary

Table 7. Effect of level of concentrate in the diet (LC: low concentrate vs. HC: high concentrate) and pH (6.4 vs. 5.6) on quantification (pg/10 ng of DNA) of *Anaerovibrio lipolytica* and *Butyrivibrio fibrisolvens* vaccenic acid (VA) and stearic acid (SA) subgroups in a composite sample of d 6 to 8

	LC		HC			*P*-value[1]		
Group	6.4	5.6	6.4	5.6	SEM	C	P	C × P
A. lipolytica	42.5[b]	0.91[c]	111.4[a]	0.48[c]	9.7	0.002	<0.001	0.002
B. fibrisolvens VA	1,314[a]	557.7[b]	1,378[a]	111.5[c]	114	0.086	<0.001	0.028
B. fibrisolvens SA	405.5	653.3	438.2	525.3	141	0.611	0.093	0.396

[a–c]When interaction is significant ($P < 0.05$), different superscripts within a row indicate means differ significantly.

[1]C = concentrate level, P = pH, and C × P = concentrate × pH interaction.

PUFA were not decreased with the HC diet at pH 6.4 (Table 5). It is possible that higher *A. lipolytica* DNA concentration is not necessarily linked to higher lipase activity. *Butyrivibrio* VA subgroup DNA concentration was reduced at pH 5.6 in both diets but this reduction was bigger in the HC than in the LC diet and its concentration at pH 6.4 was similar between diets (Table 7). This lower DNA concentration of *Butyrivibrio* VA subgroup agrees with Mosoni et al. (2007) who observed that a concentrate-supplemented diet induced a decrease (−1 log) on 3 species of cellulolytic bacteria in sheep concomitant with the rumen acidification. Moreover, FA analyses of pure strains of rumen bacteria have shown that some major cellulolytic bacteria are enriched in *iso*-$C_{14:0}$ and *iso*-$C_{15:0}$ (Minato et al., 1988). Effluent branched-chain FA mainly originate from bacteria leaving the fermenter. The concentrations of *iso*-$C_{14:0}$ and *iso*-$C_{15:0}$ in the effluent were lower at pH 5.6 than at pH 6.4 (*iso*-$C_{14:0}$: 0.06 vs. 0.25%; *iso*-$C_{15:0}$: 0.09 vs. 0.36% total FA), confirming that pH 5.6 inhibited cellulolytic bacteria. The reduction in *Butyrivibrio* VA subgroup DNA concentration at pH 5.6 is in line with the reduction in *trans*-11 $C_{18:1}$ and *cis*-9, *trans*-11 CLA proportions in the 1-h and 24-h effluents in both diets (Table 5). In addition, the HC diet had numerically lower proportions of *trans*-11 $C_{18:1}$ and *cis*-9,*trans*-11 CLA in the 1-h and 24-h effluents than the LC diet at pH 5.6 (Table 5), in line with the results observed in DNA concentration (Table 7). Results of DNA concentration in the present experiment along with the effluent FA profile results indicate that low pH inhibits some cellulolytic bacteria involved in biohydrogenation processes but this effect is more important when using high-concentrate diets.

Surprisingly, the DNA concentration of *Butyrivibrio* SA subgroup tended to be higher at pH 5.6 than at pH 6.4 (Table 7) in spite of the large decrease in $C_{18:0}$ in the 1- and 24-h effluents (Table 5). This might suggest that the *Butyrivibrio* SA subgroup is playing only a minor role in the overall $C_{18:0}$ production or its metabolic activity may not be proportional to 16S rRNA concentration. In addition, formation of $C_{18:0}$ by *Butyrivibrio* SA subgroup was through the saturation of *trans*-11 $C_{18:1}$, whereas lowered $C_{18:0}$ was associated with accumulation of *trans*-10 $C_{18:1}$. *Butyrivibrio* SA subgroup seems to be more resistent to low pH conditions compared with *Butyrivibrio* VA subgroup, as no reduction in its DNA concentration was observed in the current trial and in a previous in vitro experiment (Fuentes et al., 2008).

CONCLUSIONS

Results of the present experiment confirm that pH, not the amount of concentrate in the diet, is the main factor affecting the biohydrogenation process that results in the accumulation of *trans*-10 $C_{18:1}$ and *trans*-10,*cis*-12 CLA, fatty acids that are associated with the reduction in milk fat percentage observed in vivo. However, diets rich in concentrate also increase, but to a lesser extent, the *trans*-10,*cis*-12 CLA proportion in the effluent.

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